



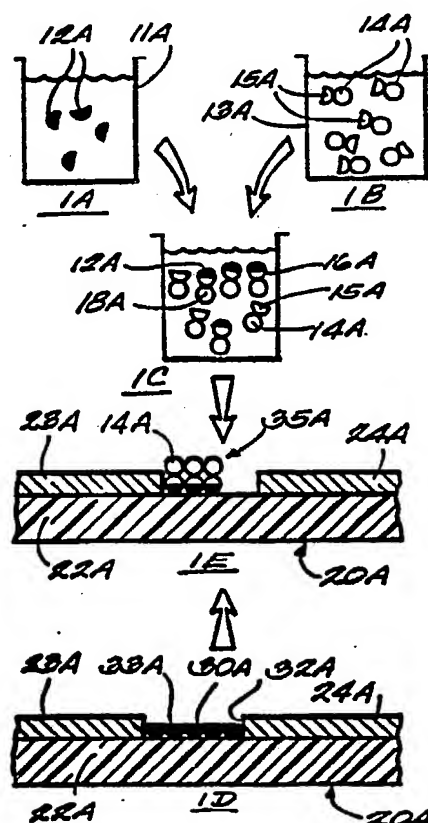
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US89/05028 (22) International Filing Date: 9 November 1989 (09.11.89) (30) Priority data: 269,569 10 November 1988 (10.11.88) US (71) Applicant: MIDWEST RESEARCH TECHNOLOGIES, INC. [US/US]; 5510 West Florist Avenue, Milwaukee, WI 53218 (US). (72) Inventors: MROCZKOWSKI, Susan, J. ; 8376 Tuckaway Shores Drive, Franklin, WI 53132 (US). SIEGESMUND, Kenneth, A. ; 17825 Primrose Lane, Brookfield, WI 53005 (US). YORDE, Donald, E. ; 5427 Tory Lane, Colgate, WI 53017 (US). (74) Agents: MEYERS, Philip, G. et al.; Foley &amp; Lardner, 777 East Wisconsin Avenue, Milwaukee, WI 53202-5367 (US).</p>	<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  Published With international search report.</p>	

(54) Title: METHOD FOR ELECTRICAL DETECTION OF A BINDING REACTION

## (57) Abstract

A method is disclosed for detecting the occurrence of a binding or complex-forming reaction between specific substances by utilizing the binding reaction to modify an electrical circuit, and then measuring a change in the electrical state of the circuit. A diagnostic element (1D) useful in such a method includes a layer of a biogenic substance (30A), such as an antigenic (12A), coated onto a non-conductive base (22A) between a pair of electrical conductors (23A, 24A) superposed on the base (22A). Electrically conductive, metallic particles (14A) having antibody (15A) which reacts with the antigen (12A) bound thereto (15A), are added to the antigen layer (30A) deposited on the base (22A) and allowed to react therewith. Electrically conductive particles (14A) are thereby bound to the base (22A) due to the binding reaction between the antigen (12A) and antibody (15A) to thereby form aggregates (35A) of electrically conductive particles (14A) which modify the circuit.



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## METHOD FOR ELECTRICAL DETECTION OF A BINDING REACTION

### TECHNICAL FIELD

This invention relates to a method for electrically detecting a binding reaction between a pair  
5 of chemical substances, particularly biogenic substances such as antigens and antibodies or nucleotides.

### BACKGROUND OF THE INVENTION

Recently several types of electrical immunoassay techniques have been developed. One such  
10 technique utilizes field effect transistors coated with a layer of antibody in the gate region. If an antigen-antibody reaction occurs, the charge concentration of the transistor changes. Examples of this type of system are given in Schenck U.S. Patent No.  
15 4,238,757, issued December 9, 1980; Guckel U.S. Patent No. 4,180,771, issued December 25, 1981; Malmros U.S. Patent Nos. 4,334,880, issued June 15, 1982 and 4,444,892, issued April 24, 1984, and Japanese Patent Publication No. 60-29658. In another system, a body  
20 fluid containing an analyte to be detected is deposited onto a surface which has been coated with a reagent that binds specifically to the analyte, so that a binding reaction takes place. A tagged reagent is then added which reacts with the analyte-reagent complex or with the  
25 reagent to change the electrical reactance of the surface. See Ebersole U.S. Patent No. 4,219,335, issued August 26, 1980.

Several other methods have been proposed for measuring immunologic reactions electrically. A  
30 voltametric immunoassay can be carried by labeling one immunoreactant with an electroactive substance. Pace U.S. Patent No. 4,233,144, issued November 11, 1980, is illustrative of one such technique. Another method involves sandwiching an antigen-antibody layer between

two conductive layers and measuring the electrical capacitance of the resulting laminate. Giaever U.S. Patent No. 4,054,646, issued October 18, 1977, describes such a method. A further type of capacitance-measuring system includes a pair of electrodes coated with a substrate and immersed in a medium containing a material which specifically binds with the substrate, as described in Arwin U.S. Patent No. 4,072,576. A further method combines change effect signal detection with an enzyme immunoassay technique. Such a method is disclosed by Gibbons U.S. Patent No. 4,287,300, issued September 1, 1981. The foregoing electrical methods have, however, failed to provide medical practitioners and laboratories with a simple, fast, sensitive, inexpensive and easy-to-use method of performing an immunodiagnostic test.

One aspect of the present invention involves the use of antigen or antibody-labeled colloidal gold particles. In general, "colloidal gold" refers to a suspension of fine gold particles in water or aqueous solution. Preparation of such particles is disclosed by DeMey, et al. U.S. Patent No. 4,446,238, issued May 1, 1984, and DeMey, et al. U.S. Patent No. 4,420,558, issued December 13, 1983. The entire contents of both such DeMey patents are incorporated herein by reference. Such colloidal gold preparations have been previously used in immunodiagnostic tests wherein the results are determined optically by observing small amounts of light reflected as a result of the antigen-antibody reaction. The foregoing patents to DeMey disclose a bright field light method of the foregoing type. Silver enhancement has been previously used as a means for staining gold particles. The present invention advantageously employs colloidal gold, optionally with silver enhancement, in a new immunodiagnostic method.

### SUMMARY OF THE INVENTION

The present invention provides a method for detecting a binding reaction between a pair of first and second substances, particularly biogenic substances, which specifically bind together. The method of the invention involves bringing the substances together so that the binding reaction between them causes full or partial completion (closing) of an essentially open electrical circuit. The resulting change in the electrical state of the circuit indicates the binding reaction.

According to a further aspect of the invention, a diagnostic element for use in detecting a binding reaction comprises a pair of spaced-apart, electrical conductors, particularly conductive layers, disposed on a substantially non-electrically conductive base. The base may comprise a support having a layer formed thereon which has a high affinity for protein binding and has a moderate to high resistance in comparison to the conductors. The space between the conductors defines a path or channel. One of a pair of substances which bind to each other is deposited on and affixed to the binding layer of the non-conductive base between the conductors, such as on the bottom wall of a channel. Means forming an electrical circuit is connected to each of the conductors so that the channel constitutes a break in the circuit.

As used herein, the term "diagnostic element" refers to the base, conductors, and layer of one of the binding substances, without the means defining the electrical circuit. Such a diagnostic element and means forming an electrical circuit can readily be used in conjunction with any suitable means for fully or partially bridging the break in the circuit due to the binding reaction between the pair of substances. One such means involves adhering one of the substances to the surfaces of electrically conductive particles.

**BRIEF DESCRIPTION OF THE DRAWING**

Preferred exemplary embodiments will hereafter be described in conjunction with the appended drawing, wherein like designations denote like elements, and:

5           Figures 1A, 1B, 1C, 1D and 1E and Figures 2A, 2B, 2C, 2D and 2E are schematic diagrams illustrating an immunodiagnostic method according to the invention;

          Figure 3 is a schematic diagram illustrating a  
10          reaction detector according to one embodiment of the invention;

          Figures 4A and 4B are schematic diagrams showing aggregate formation according to the method of Figures 1A-1E and 2A-2E, respectively;

          Figure 5 is a schematic diagram showing binding  
15          of a conductive particle to a non-conductive base according to one embodiment of the invention;

          Figure 6 is a cross-sectional view of a diagnostic element according to one embodiment of the invention;

20          Figure 7 is a cross-sectional view of an alternative diagnostic element according to the invention;

          Figure 8 is a plan view of a multiple diagnostic element according to the invention;

25          Figure 9 is a plan view of an alternative multiple diagnostic element according to the invention;

          Figure 10 is a schematic diagram of an uncoated bound aggregate according to the invention;

30          Figure 11 is a schematic diagram of a coated bound aggregate according to the invention;

          Figure 12 is a schematic diagram of resistive shunting of current according to the invention;

35          Figure 13 is a graph wherein resistance values after silver overcoating and silver counts, both as obtained in Example 3, are plotted against gold-antibody conjugate dilution; and

Figure 14 is a graph wherein percent of maximum resistance determined in Example 4 is plotted against amount of competing antigen.

#### DETAILED DESCRIPTION

5           The method of the invention is particularly useful for detecting antigens in the fluids or tissues of humans or animals. Such antigens include drugs, toxins, hormones, allergens, tumor markers, factors, enzymes, steroids, nucleotides and other substances as listed in  
10   Huang U.S. Patent No. 4,327,073, issued April 27, 1982, the entire content of which is incorporated herein by reference. Any of the foregoing listed substances can provoke the production of a reactive substance (antibody) which reacts with and binds to the antigen. Accordingly,  
15   the method of the present invention is useful for detection of a wide variety of substances which may be present in the living body of a human or lower animal, for example, in drug overdose treatment, where it is desired to quickly determine which drug a patient has  
20   taken.

          Figures 1A-1E schematically illustrate a method for detecting an antigen according to the present invention. Referring to Figures 1A through 1C, a patient sample 11A (FIG 1A) such as whole blood, blood  
25   serum or urine, containing a particular antigen 12A is mixed with a colloidal gold preparation 13A (FIG. 1B) containing a predetermined amount of gold particles 14A having antibodies 15A fixed to the outer surfaces thereof. Antibodies 15A specifically bind to antigen 12A  
30   (FIG. 1C). In the resulting mixture 16A, antigen 12A binds with available antibodies 15A, resulting in free complexes 18A, comprising both antigen 12A and antibody 15A bound to particles 14A. Since there are more antibodies 15A than antigens 12A, some antibodies 15A  
35   remain free, i.e., unbound to an antigen 12A.

As illustrated in Figures 2A-2C, the foregoing procedure is also carried out using a control sample 11B (FIG. 2) lacking the antigen 12A and a second colloidal gold preparation 13B (FIG. 2B) substantially identical in composition to preparation 13A used with patient sample 11A. The thus-formed second mixture 16B (FIG. 2C) lacks the complexes 18A shown in Figure 1A, and correspondingly has a greater number of particles 14B having unbound antigen 15B on the surfaces thereof. The first mixture 16A, corresponding to the patient sample (FIG. 1C), and the second mixture 16B (FIG. 2C), corresponding to the control, are then ready for use with a reaction detector 20 according to the invention (FIGS. 1D, 2D).

Referring now to Figure 3, reaction detector 20 includes a non-electrically conductive base 22, a pair of thin, spaced-apart electrically conductive layers 23, 24 disposed side-by-side on base 22 with a channel 32 formed therebetween, and means defining an electrical circuit, such as an ohmmeter 26 functionally connected to layers 23, 24 as shown by means such as wires 28. Layers 23, 24 act as a pair of positive and negative terminals for the circuit.

Referring again to Figures 1 and 2, identical first and second reaction detectors 20A (FIG. 1D) and 20B (FIG. 2D) are prepared in advance for use with mixtures 16A, 16B, respectively. Samples of antigen in a carrier liquid (e.g., water or saline solution) are poured into shallow channels or grooves 32A, 32B defined between layers 23A,B and 24A,B to cause antigen to bind to the surfaces of bottom walls 33A,B of channels 32A,B to form antigen layers 30A, 30B (FIGS. 1D, 2D). These antigen layers 30A, 30B are made of the same type of antigen as antigen 12A to be detected.

Referring now to Figure 1E, first mixture 16A (FIG. 1C), corresponding to patient sample 11A (FIG. 1A), is poured into channel 32A of first detector 20A to cause binding of antigen layer 30A and antibody 15A. The foregoing procedure is also carried out using the control



5 mixture 16B (lacking complexes 18A) and second detector 20B (FIG. 2E). Conductive particles 14A having free antibodies 15A thereon effectively become bound to bottom wall 33A via antigen layer 30A due to the antigen-antibody binding reaction. Complexes 18A containing antigen 12A from sample 11A do not tend to become bound to bottom wall 33A.

10 After a suitable time to allow the antigen-antibody interaction to take place, channel 32A is flushed with a suitable liquid, e.g., water or saline solution, to wash away any unbound particles 14A, then dried by any suitable means, such as heating or allowing the reaction detector to stand open to the air. The resistance measurement can also be performed wet, without  
15 any drying step. Even when wet, the difference in measured resistance is sufficient to indicate whether the binding reaction has occurred.

Non-specific binding of particles to layers 30A, 30B occurs to some extent. Ordinary washing  
20 procedures may not be sufficient to remove such non-specifically bound particles from the antigen layer. However, it has been found that ultrasonic treatment of the samples at a low level can remove non-specifically bound particles without removing specifically bound  
25 particles, i.e., particles bound due to antigen-antibody binding involving the substance being detected for. This is particularly important as a means of preventing a false positive result. Such a false positive result is a decrease in resistance due to non-specifically bound  
30 particles, rather than specifically bound particles.

Referring now to Figures 4A and 4B, Figure 4A corresponds to the same state as Figure 1E, and Figure 4B corresponds to the same state as Figure 2E. Figures 4A, 4B illustrate the differences in the extent of the  
35 binding reaction not apparent in Figures 1E, 2E. Ohmmeters 26A and 26B register the resistance across channels 32A and 32B for each of the detectors 20A and 20B. For the control (FIG. 4B), all particles 14B having

free antibodies 15B deposited thereon are available for binding with antigen layer 30B bound to bottom wall 33B. As a result, complexes 35B are formed at bottom wall 33B and are anchored thereto. As illustrated in Figure 4B, complexes 35B tend to cluster together in contact with each other to form aggregates or chains 39B which effectively bridge channel 32B. Since particles 14B are electrically conductive, aggregates 39B effectively provide an electrical connection between layers 23B and 24B, completing an electrical circuit defined by ohmmeter 26B, wires 28B, layers 23B, 24B, and aggregates 39B. This is reflected by the resistance reading given by ohmmeter 26B. A drastic decrease in resistance occurs as a result of bridging of aggregates 39B.

The reaction for the mixture 16A corresponding to the patient sample proceeds in a similar fashion, except that this mixture 16A already contains complexes 18A formed by reaction of antigen 12A with antibody 15A. Since the antibodies of these complexes 18A are already bound with at least some antigen 12A, these complexes 18A do not tend to bind to the layer of antigen 30A at bottom wall 33A. In mixture 16A, the number of free antibodies 15A deposited on particles 14A is less than in mixture 16B, since some of these antibodies 15A were used to form complexes 18A. As shown in Figure 4A, aggregates 39A form, but there are fewer such aggregates, and correspondingly less bridging of channel 32A. As a result, the decrease in resistance registered by ohmmeter 26A, if any, is less than the decrease in resistance registered by ohmmeter 26B. This difference in readings indicates the presence of antigen 12A in the patient sample 11A. If patient sample 11A does not contain any antigen 12A, then the decrease in resistance for reaction detector 20A would be the same as the decrease in resistance for detector 20B.

If resistance values corresponding to specific antigen levels in the sample are well known for a specific test, the foregoing procedure can be carried out

without the control illustrated in Figures 2A-2E, 4B. However, the use of a control is preferred because the comparative resistance readings produced by the control afford more accurate results.

5           The procedure shown in Figures 1 through 4 is greatly simplified for purposes of illustration. Conductive particles 14A,B are larger than antigen 12A and antibodies 15A,B. A number of antibodies 15A,B are bound to a single conductive particle 14A or 14B, and  
10           similarly a number of antigens 12A can bind with antibodies 15A on the surface of a single particle 14A or 14B. Figure 5 schematically illustrates how a conductive particle 14B having antibodies 15B on its surface becomes bound to bottom wall 33B via antigen layer 30B.

15           The method of Figures 1-4 utilizes a positive control, that is, a decrease in resistance due to bridging of the channel by the conductive particles occurs for the control sample, not the unknown (patient) sample. The method of the invention may also be carried  
20           out using a negative control wherein the sample containing the unknown, for example, an antibody, is reacted directly with the gold particles, and then the gold particles are brought in contact with the reaction surface coated with the corresponding antigen. The  
25           procedure is substantially the same as shown in Figure 2B, except that the amount of the antibody is unknown.

          Many variations of the method of the invention, including both competitive and non-competitive procedures, are possible. The process illustrated in  
30           Figs. 1A-1E is a competitive reaction wherein the antigen is bound to the diagnostic element, and free antigen in the sample 11A competes with the bound antigen for sites on the conductively labelled antibody. The preincubation step shown in Fig. 1C may be omitted, and  
35           the sample 11A and colloidal gold 13A may be added directly to antigen layer 30A bound to the diagnostic element. Alternatively, the antibody may instead be bound to the diagnostic element, and conductively

labelled antigen can compete with free antigen in the sample for antibody binding sites. In either case, the resistance measurements are directly related to the amount of free antigen in the sample, in other words, the measured resistance increases directly with increasing amounts of free antigen in the sample.

In a non-competitive variation useful for antigens that can bind to more than one antibody at the same time, a first antibody is bound to the diagnostic element in a predetermined amount in excess of the amount required to bind all available free antigen in the sample. The sample is added to the bound antibody and allowed to react. A second, conductively labelled antibody is added, either later or at the same time as the sample. The second antibody also reacts with the antigen, resulting in a complex comprising first antibody-antigen-second antibody-conductive particle bound to the diagnostic element. The two binding sites on the antigen may be identical, structurally different, or two-site immunometric. The resulting resistance measurements are inversely related to the amounts of free antigen in the sample, such that the resistance decreases as the amount of free antigen increases.

Another non-competitive variation is useful for determining amounts of specific antibody in a sample, particularly an antibody titer for a disease or allergy. The antigen is bound to the diagnostic element in sufficient excess to bind to the antibody in proportion to its concentration. A sample containing free antibody is added to the bound antigen and allowed to react. A secondary antibody carrying the conductive particles is added, either later or at the same time as the sample. The secondary antibody reacts with the first antibody, i.e., treats it as an antigen, forming a complex comprising antigen-first antibody-second antibody-conductive particle. The secondary antibody could be, for example, anti-immunoglobulin G or E. The resulting resistance measurements are inversely related

to the amount of free (first) antibody in the sample. A secondary antibody can also be used in embodiments wherein resistance change is directly related to measured antigen in the sample in order to increase sensitivity.

5 Bases 22A, 22B discussed above must have a highly bioreactive surface. "Bioreactive" as discussed herein refers to the ability of the surface to bind biogenic substances such as proteins or nucleotides. Bioreactivity values measured for various organic and  
10 inorganic substances vary widely even among chemically similar substances. However, certain plastics and metal oxides and nitrides are generally bioreactive. In a series of enzyme immunoassay experiments (see Example 3) the absorption of light at 490 nanometers was used as a  
15 standard for determining binding by a rabbit IgG-goat anti-rabbit IgG complex to each surface tested. Nylon 66 had the highest level of bioreactivity measured. For purposes of the present invention, substances having a bioreactivity at least 70% that of Nylon 66 for proteins  
20 are considered highly bioreactive. Compounds having about 50-70% as much bioreactivity as Nylon 66 are mildly bioreactive, compounds having 10-50% are somewhat bioreactive, and compositions having a bioreactivity of about 10% or less are bioinert.

25 It has been found that the following substances are highly bioreactive with proteins: Nylon 66, polypropylene, mylar, chromium oxide, phenolic plastic, polystyrene, and vinyl. Chromium oxide is unusually high in protein bioreactivity for a metal oxide. Moderately  
30 bioreactive substances for proteins include chromium, titanium oxynitride, nickel oxide, tantalum nitride and carbon. Poorly bioreactive substances for proteins include titanium oxide, boron nitride, and silicon oxide. Bioinert substances for proteins include silicon nitride,  
35 barium titanium oxide, indium tin oxide, aluminum oxide and glass.

Figures 6 and 7 illustrate two diagnostic elements 10A, 10B that can be used in the method of the invention. In Figure 7, diagnostic element 10A according to the invention includes a support 40 such as a glass plate, e.g. a microscope slide, coated with a thin layer 45A of an electrically resistive, bioreactive substance such as chromium oxide. A pair of conductive layers, such as chromium metal layers 51, 52, are superposed on the resistive, bioreactive layer 45A. A channel 42 separates layers 51, 52. A layer of an antigen 53 spans channel 42, and may also cover layers 51, 52. Layers 45A, 51, 52 are preferably formed by sputtering on a glass plate. Channel 42 is then formed by photolithography using an etchant that selectively attacks the conductive layer but does not attack layer 45A. In this diagnostic element, the width of channel 42 relative to the size of the conductive particles to be used is important because the binding reaction is primarily indicated by complete bridging of channel 42 by aggregates of the conductive particles. The resistive layer 45A is highly resistive, i.e. more than about  $10^6$  ohmS-cm, so that virtually no change in resistance is caused by partial bridging as shown in Figure 4A.

Figure 6 illustrates an alternative diagnostic element wherein conductive layers 51, 52 are formed directly on support 40, with channel 42 formed therebetween. Resistive layer 45B is superposed over support 40 and layers 51, 52, and antigen layer 53 is then formed thereon. In this embodiment layer 45B is only moderately resistive, e.g.  $10^3$ - $10^6$  ohmS-cm, so that a substantial change in resistance will occur due to resistive shunting of current through aggregates bound to layer 53, even when no complete bridging occurs.

Figure 12 illustrates a resistor through which resistive shunting occurs. In seeking the path 71 of least resistance, the current will be shunted through bound aggregates 72 which offer a lower resistance than the support 40. The overall resistance drop across

channel 42 will depend on how many aggregates are bound, and can provide a quantitative indication of the amount of binding being measured. In the resistor shown, the calculated resistance drops from 70,000 to 40,030 ohms following the reaction.

The width of channel 32 or 42 may vary, particularly in relation to the simple numerical average diameter of the conductive particles forming a chain to bridge the channel. The following table states preferred ranges for dimensions for gap-bridging embodiments according to the present invention:

TABLE 1

15	Average Particle Diameter (Microns)	Channel Width (Microns)	Ratio of Channel Width to Particle Diameter
	0.01-500	0.1-20,000	5:1 to 40:1
	0.01-10	0.1-100	10:1 to 30:1
	0.01-1	1-25	15:1 to 25:1

20 A 20:1 ratio of channel width to average particle diameter is typical, e.g., the channel has a width of 10 microns, and the average diameter of the conductive particles is 0.5 microns. The foregoing ranges are also useful in resistive shunting embodiments, but much  
25 larger channel widths may be employed, such as up to 1 mm, 1 cm, or greater, depending on the desired application.

As an alternative to a channel, diagnostic elements according to the invention, especially resistive shunting embodiments, may utilize a resistive,  
30 bioreactive path other than a channel. Such a path may comprise, for example, a curved line which spans the conductors but does not represent the shortest distance between them. Similarly, the conductors need not be in

the form of layers. Small wires superposed on the base can, for example, be employed as the conductors.

Proteins have an affinity for materials such as polystyrene, chromium oxide and the like, and tend to become bound thereto under suitable conditions. Proteins can also readily become bound to the surfaces of fine metal particles, such as gold particles, using the procedure described below. In embodiments of the invention which involve antigen-antibody binding, it is preferred to bind the antigen to the bottom wall of the channel and bind the antibodies to the conductive particles. However, the reverse arrangement (antigen-particles, antibody-channel) can also be employed.

Conductive layers 51, 52 may have any desired dimensions which prove functional. To reduce the size of the diagnostic element, these layers are generally as thin as possible, and preferably have a thickness no greater than about 5 microns, preferably no greater than about 0.5 microns, particularly a thickness in the range of 0.001-0.005 microns. Conventional sputter deposition can be readily used to form the conductive layers in any desired shape.

Layers 51, 52 (or 23, 24) may be formed of any suitable conductive material, particularly an electrically conductive metal such as gold, silver, platinum, copper, chromium or aluminum. Particles 14A, 14B are preferably made from a conductive metal such as gold, silver, or platinum, and may also be made of carbon platelets or plastic particles having a conductive metal coating, especially gold-coated polystyrene spheres. Such coated spheres are lighter than comparable solid metal spheres and are thus better able to maintain bonding to the surface.

The extent of binding of an antibody to metal particles is influenced by various factors. A series of enzyme immunoassay experiments demonstrated that affinity-purified antibody tends to bind to gold particles to a much greater extent than impure, whole



serum antibody, and that the pH of the system also had a strong effect. A slightly acidic pH (e.g. 6-7) produced several-fold greater binding than a basic pH of 9-10 for large (greater than 0.5  $\mu$ m diameter) particles.

5 Polyethylene glycol treatment of the gold particles may also be used if needed to stabilize large gold particles; see Horisberger et al., J. Histochem. Cytochem., 25:295-305 (1977).

10 The antibody concentration on the gold particles should be high enough to allow the antigen-antibody reaction to occur on the surfaces of the gold particles to an extent sufficient for detection by the method of the invention. On the other hand, if the antibody concentration is too great, the antibody layer  
15 may have an insulating effect which will result in a false negative result, i.e., will block the drop in resistance that would normally occur, unless an additional step of overcoating the aggregates with a conductive metal is used, as described hereafter.

20 Base 22 described above is made of a bioreactive plastic, preferably polypropylene, mylar, phenolic plastic, vinyl, methyl cellulose, nylon or polystyrene. The polarity of protein molecules causes such molecules to bind to such a plastic base to form a  
25 substantially complete, homogenous coating of antigen. Glass by itself is not generally employed as the base (support) since antigens have a poor affinity for a glass surface and it has proved difficult to adhere a layer of antigen to a glass slide. However, according to a  
30 further aspect of the invention, it has been found that a glass slide can be surface treated so that antigen affinity for the coating on the glass slide becomes as great or greater than antigen affinity for a conventional polystyrene slide. As noted above, this surface  
35 treatment comprises coating the glass support 40 with a thin layer 45A or 45B of a material having a moderate to high electrical resistance as compared to the conductive layers. The resistance of this layer is in the range

10<sup>3</sup>-10<sup>8</sup> ohms-cm, preferably 10<sup>4</sup>-10<sup>7</sup> ohms-cm, as compared to a resistance of less than 100 ohms-cm for the conductors. Moderately resistive materials for purposes of the invention are those having measured resistances in the range of 10<sup>3</sup> up to 10<sup>6</sup> ohms-cm when deposited on a glass support, whereas highly resistive materials have comparable measured resistances of 10<sup>6</sup> or more.

Bioreactive substances suitable for forming layer 45A or 45B include carbon, hydrophilic organic polymers, and inorganic metal oxides and nitrides. Such inorganic metal compounds include oxides, nitrides and oxynitrides of boron, aluminum, silicon, cadmium, copper, nickel, cobalt, iron, manganese, and metals of Groups IIA, IIIB through VIB. Especially preferred inorganic metal compounds include chromium oxide (CrO<sub>3</sub>), titanium oxynitride (TiO<sub>x</sub>N<sub>y</sub>), tantalum nitride, cermet materials such as chromium/silicon oxide (Cr·SiO) or gold/silicon oxide, and other inorganic materials currently used in resistors. Hydrophilic organic polymers include well-known plastics such as mylar, polystyrene and nylons, such as Nylon 66.

If chromium oxide is used as layer 45A, it is usually deposited in the form of CrO<sub>2</sub>. To make it more bioreactive, the layer may be aged in the presence of oxygen and water vapor to convert it to CrO<sub>3</sub>, which is hydrophilic. This aging can be accelerated by placing the coated base or finished diagnostic element in a humidity chamber. Carbon in any convenient form, e.g. graphite, is a theoretically ideal material for layer 45 in view of its biocompatibility, although its thin film resistance may be hard to control within narrow ranges.

Cermet resistor materials have a high resistance, are stable, and have low negative temperature coefficients, such that the diagnostic element will not be excessively temperature sensitive. When heated in air, cermet films oxidize and increase in resistance, and are usually provided with a protective overcoating to

prevent oxidation and/or hydration. In the present invention, however, layer 45 does not require such a protective coating.

Figure 8 illustrates a further embodiment of the diagnostic element of the invention wherein a multiplicity of reaction sites are disposed on a single non-conductive base 22. Each reaction site comprises a pair of conductors, such as layers 23, 24, having a layer of a biogenic substance which undergoes specific binding disposed in the channel or path therebetween. The biogenic substance, such as an antigen, may be the same or different for each reaction site. Conductive means 28 for this embodiment comprises a series of individual electrical conductors 61 which each connect to a common conductor 62, which in turn connects to a terminal plate 63 mounted on the edge of base 22. Pairs of layers 23, 24 are arrayed in rows and columns on base 22.

In the embodiment of Figure 8, rows of plates 63 are disposed on adjacent sides of rectangular base 22. To prevent overlapping between a first set 66 of conductors 62 connected to layers 23 and a second set 67 of conductors 62 connected to layers 24, all but one of conductors 62 of second set 67 are located on the reverse side of base 22. These conductors 62 are illustrated by broken lines in Figure 8. Outer conductor 62A does not cross any conductors 62 of first set 66 and thus does not need to be on the other side of base 22, although it can be so located if desired. Individual conductors 61 connected to common conductors 62 of second set 67 on the reverse side of base 22 include portions 69 which extend through the thickness of base 22 and connect with such conductors 62, shown by broken lines in Fig. 8.

Ohmmeter 26 can be connected to various combinations of terminal plates 63 to measure resistance for each pair of layers 23, 24. To make such a measurement, ohmmeter 26 is connected to two plates 63 on different edges of base 22. Connecting an ohmmeter to plates 63C, 63D would test the indicated pair of layers

23C, 24D. This embodiment allows a single diagnostic element according to the invention to test a single patient sample for a number of different substances, such as antigens, since each pair of layers 23, 24 can have a different substance bound therebetween, or have no substance bound therebetween so as to provide a control. In the alternative, the same substance may be tested for a number of times to provide a more certain result.

In an alternative embodiment shown in Figure 9, terminals 63 are disposed along only one edge of base 22. One terminal 63E is connected to each layer 23 by a single, multibranched conductor 62E. A series of terminals 63F are each connected to one of layers 24 by separate conductors 62F. Conductors 62E,F are arranged to not cross each other, so that all of conductors 62E,F are disposed on the front face of the diagnostic element as shown. For this purpose terminal 63E is located at one end of the row of spaced-apart terminals 63E,F. This embodiment avoids the need to provide wires, conductive lines, or the like on both sides of the plate, and allows the terminals to be disposed on a common edge.

Resistive shunting according to the method of the invention may be greatly enhanced by overcoating the aggregates with a layer of a conductive substance, particularly a metal such as silver, gold, or platinum. The applied coating must stick selectively to the aggregates but not to the remainder of the the path between the conductors. If gold particles are used as the particles to which one of the binding substances are bound, silver enhancement may be used to form a conductive silver coating over the aggregates.

Conductive metal overcoating is especially useful for resistive shunting embodiments of the invention for two reasons. First, it can eliminate problems with junction resistance effects. Referring to Figure 10, resistive shunting is minimized by the relatively small contact surface of the aggregates 76 with the underlying layer 53. In addition, the biogenic

coating 77 on the gold particles 78 can form an insulating barrier such that the junction resistance  $R_j$  is high enough to prevent resistive shunting. By contrast, the conductive metal coating 79 shown in Figure 11 creates a much lower resistance  $R_j$  and allows resistive shunting to occur. This embodiment of the invention is especially preferred because it improves reliability, as demonstrated by the results of Example 2 below.

Second, metal overcoating can eliminate the need to use particles which are both bioreactive and conductive. Since the metal overcoating can conduct the current, the particles can be made of a non-conductive substance, e.g., non-conductive beads may be used so long as the metal overcoating sticks selectively thereto. This advantage also pertains to gap bridging embodiments as well.

The following examples describe a gap bridging embodiment (Example 1), a resistive shunting embodiment (Example 2) and an example of a procedure for determining bioreactivity according to the invention (Example 3).

#### EXAMPLE 1

##### A. Preparation of Colloidal Gold

The following procedure was used to make a suspension of gold particles. Glassware including a flask and stirring bar were cleaned by sonication in a bath sonicator, first in ethanol, then acetone, then petroleum ether, for about 5 minutes each. The glassware was then blown dry with a Freon refrigerant, then rinsed twice with double glass-distilled water. About 1 ml (milliliter) of a 1% w/v (weight/volume) filtered solution of gold chloride in water and 99 ml of filtered, double glass distilled water were added to the cleaned flask. The resulting mixture was then heated and stirred with the cleaned stirring bar until boiling. At that time 300  $\mu$ l of a 1% w/v filtered solution of trisodium

citrate in water was added. Boiling was continued with stirring, and additional 1% trisodium citrate solution was added in amounts of 100, 50 and 50  $\mu$ l at intervals of 5, 12 and 15 minutes, respectively, from the time the mixture began boiling. After 30 minutes of boiling the flask was removed from heat and allowed to cool. The average particle size of the resulting colloidal gold particles was 100 nm as determined using a transmission electron microscope. The flask containing the colloidal gold preparation was sealed and stored at 4°C.

#### B. Preparation of Gold-Labelled Proteins

About 9 ml of the colloidal gold preparation made in part A above, 100  $\mu$ l of 0.2 M potassium carbonate and 20  $\mu$ l of a protein, an affinity-purified goat anti-rabbit immunoglobulin (IgG) antibody, were added to a 15 ml conical centrifuge tube. The ingredients were mixed overnight on a tube rotator. 1 ml of a filtered aqueous polyethylene glycol solution (2 mg PEG/ml) was then added as a stabilizing agent. Filtration in this and subsequent procedures was carried out using a 0.2 micron filter disk fitted into a syringe. The resulting gold-protein conjugates were concentrated by centrifugation at 11,000 rpm for 10 minutes. The supernatant was discarded, and the resulting pellet of gold particles was pipetted into a 1.5 ml centrifuge tube. The particles were then washed by centrifugation four times with a phosphate buffered saline solution (0.01 M sodium phosphate dibasic, 0.15 M sodium chloride, 0.1 mg/ml polyethylene glycol, pH 7.4) containing 0.2% sodium azide as a preservative to remove unbound protein. The conjugated gold particles were then resuspended in 1.4 ml of phosphate buffered saline solution containing 0.2% sodium azide.

### C. Preparation of Diagnostic Element

A glass slide was coated with a thin layer of chromium oxide ( $\text{CrO}_x$ , wherein  $x=1-3$ ) by radiofrequency (rf) magnetron sputter deposition using a Materials Research Corporation Model 822 Sputtersphere. A chromium plate target was used as the cathode. The glass slide was placed on a copper plate anode disposed beneath the cathode. Deposition was carried out under the following conditions: system base pressure  $3 \times 10^{-7}$  Torr, total pressure  $8 \times 10^{-3}$  Torr, forward power 1,000W, target voltage 335V, reflected power 0 watts, cathode to anode distance  $2\frac{1}{2}$  inches, and a sputtering time of 30 minutes. The system was backfilled with oxygen. The desired chromium oxide layer having a thickness of about  $0.1 \mu\text{m}$  was thereby formed by deposition in the oxygen plasma. A layer of chromium was then deposited over the chromium oxide layer by repeating the foregoing procedure in an inert argon plasma, resulting in a surface layer of chromium having a thickness of  $1 \mu\text{m}$ .

### D. Photolithography

A line having a width of  $5 \mu\text{m}$  was then formed in the surface chromium layer of the double-coated slide of part C by photolithography. In yellow room light, a layer of photoresist plastic was first formed over the chromium layer as follows. The twice-coated slide was placed in the chuck of a Headway Model EC101 spinner under vacuum. About 3 ml of Shipley 111S photoresist was applied over the chromium layer and allowed to spread for 10 seconds. The slide was then spun at 2000 rpm for 30 seconds to form a uniform  $1 \mu\text{m}$  thick photoresist coating. Several such photoresist-coated slides were then baked in a hot plate oven on a rack for about 20 minutes at  $80^\circ\text{C}$ , then allowed to cool for 5 minutes.

A Suss MJB55 photolithographic mask aligner was calibrated and prepared for use. A mask defining a  $5 \mu\text{m}$  line was cleaned thoroughly with dissolved soap and water, then dried with a nitrogen stream that left no

residue. The mask was then placed in a mask holder under vacuum, and the mask holder was then placed in position on the aligner. The triple-coated slide was placed beneath the 5  $\mu$ m line of the mask. The mask holder was then locked in place, and the aligner was used to expose the photoresist layer to UV light for 18 seconds. The exposed slide was then removed from the aligner.

The slides were then immersed in a fresh 4:1 developer solution (4 parts water to 1 part Shipley 303A developer) for 2 minutes with no agitation, and then promptly rinsed with distilled water. Each slide was then immersed in the etchant, a solution heated to 45°C consisting of 90.8 g  $\text{AlCl}_3$ , 27.0 g  $\text{ZnCl}_2$ , 6 ml  $\text{H}_3\text{PO}_4$  and 80 ml distilled water, for about 10 seconds. During this time the etchant removed undeveloped photoresist material and chromium metal along the 5 micron line which was previously exposed to UV light. However, the etchant did not attack the chromium oxide layer. After etching, the slide was promptly rinsed in distilled water. The photoresist material was then removed from the surface of the chromium layers by immersing the slides for about 5 minutes in Shipley 1112A remover. Thereafter the slide was rinsed thoroughly in tap water, then rinsed ultrasonically in distilled water for 10 minutes. The slide was then re-immersed in the remover, and the rinsing steps were repeated to ensure that the photoresist material was completely removed.

#### E. Measurement of Binding Reaction

The resistance of the diagnostic elements prepared in part D was measured using an ohmmeter. Each element was then placed in a test tube and rinsed with a coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, 0.003 M sodium azide, pH 9.8). Pairs of diagnostic elements were prepared by adding either rabbit immunoglobulin (IgG) or bovine serum albumin (BSA) to each test tube. The elements were incubated by allowing the tubes to stand overnight at room temperature. The



protein-coated elements were then washed three times with a PBS-Triton buffer (phosphate buffered saline solution as described above containing 0.1 vol. % Triton X-100 as a wetting agent) to remove excess unbound protein. The coated substrates were then placed on a piece of parafilm in a petri dish. The colloidal gold-protein conjugate was then applied to each element in an amount sufficient to cover the upper surface of each element, and the elements were allowed to incubate (stand) for 20 minutes at room temperature. Each element was then washed by aspiration once in PBS-Triton buffer and once in distilled water, and then allowed to air dry for 1 hour. The resistance of each element was then measured using the same ohmmeter as initially used to measure the resistance of each element.

All elements tested had a starting resistance greater than 2 million ohms. Positive results were obtained for samples at line widths of 3, 5, 6 and 7.5  $\mu\text{m}$ . For these samples, the measured resistance at the second measurement, following the reaction, dropped to values ranging from 85 to 700 ohms. A few IgG samples resulted in weakly positive results ranging from 80,000 to 800,000 ohms. Negative results were obtained for the BSA samples (the resistance after the reaction remained higher than 2 million ohms), except that two weakly positive results for BSA samples were obtained at the 5  $\mu\text{m}$  line size.

Similar results were obtained when the foregoing procedure was repeated at different temperatures or incubation times. Usually, a positive result was obtained with a reaction time of 20 minutes. These variations of the procedure indicate that the reaction time can be shortened substantially.

## EXAMPLE 2

### A. Preparation of protein-labelled colloidal gold and diagnostic elements

Protein labelled colloidal gold was prepared as described in Example 1, parts A and B. A diagnostic element according to the invention was then prepared by first coating a glass microscope slide with a thin layer of chromium by rf magnetron sputter deposition using the procedure of Example 1, part C, except that no  
5 intervening layer of chromium oxide was formed. The chromium-coated slide was then subjected to photolithography as described in Example 1, part D, except that the line formed had a width of 1 millimeter.  
10

A thin layer of carbon was then deposited over the entire upper surface of the slide, as shown in Figure 6, by sputter deposition according to Example 1, part C, except as follows: the backfill gas was argon, and the target (cathode) was a graphite disk 8 inches in diameter and 0.25 inch thick. The graphite disk was  
15 epoxyed to a water-cooled copper backing plate. Deposition was carried out at 145 V with a forward power of 200 W. The desired carbon layer having a thickness of about 0.25  $\mu\text{m}$  was thereby formed by deposition in the argon plasma.  
20

### 25 B. Measurement of Immunologic Reaction

The resistance of the diagnostic elements prepared in part A was measured using an ohmmeter. Each element was then placed in a test tube and rinsed with a coating buffer (pH 9.8, as described above). Pairs of  
30 positive and negative diagnostic elements were prepared by adding either rabbit immunoglobulin (IgG) or bovine serum albumin (BSA) to each test tube. The elements were incubated by allowing the tubes to stand overnight at room temperature. The protein-coated elements were then  
35 washed three times with a PBS-Triton buffer (phosphate buffered saline solution as described above containing 0.1 vol. % Triton X-100 as a wetting agent) to remove

excess unbound protein. The coated substates were then placed on a piece of parafilm in a petri dish. The colloidal gold-protein conjugate was then applied to each element in an amount sufficient to cover the upper  
5 surface of each element, and the elements were allowed to incubate (stand) for 2, 1 or  $\frac{1}{2}$  hour at room temperature. Each element was then washed once in PBS-Triton buffer and once in distilled water, and then allowed to air dry for 1 hour. The resistance of each element was then  
10 measured using the same ohmmeter as initially used to measure the resistance of each element.

### C. Silver Enhancement

The elements prepared in part B above were rewashed with distilled water to remove chloride ions  
15 that might react with the silver enhancement reagents. A 2 M sodium citrate buffer solution, a 0.5 M hydroquinone solution and a 0.03 M silver lactate solution were prepared in a darkened room. The three reagents were then mixed together to provide 25 ml of overcoating  
20 reagent. The elements were completely immersed in this reagent for 2-3 minutes, then immersed in a 1 % acetic acid solution for 2 minutes, and then immersed in a fixative solution for for 2 minutes. The fixative used was Kodak Rapid Fix, containing ammonium thiosulfate,  
25 acetic acid, sodium metabisulfite, sodium tetraborate, and aluminum sulfate. The elements were rinsed in distilled water for 10-15 minutes and allowed to air dry. The resistance of each element was then measured using the same ohmmeter as used in Example 2, part B.

30 The resistance results (R) are summarized in Table 2. In part B, Samples 6A, 6B were incubated for 1 hour, Samples 7A, 7B were incubated for  $\frac{1}{2}$  hour, and the remaining samples were incubated for 2 hours.

TABLE 2

		Starting R	R After Gold	R After Silver	%
	<u>Sample</u>	<u>(Ohms)</u>	<u>(Ohms)</u>	<u>Enh. (Ohms)</u>	<u>Change</u>
5	1A (BSA)	298,000	394,000	304,000	+0.002
	1B (IgG)	357,000	444,000	379	-99.9
	2A (BSA)	1,060,000	1,280,000	950,000	-10.0
	2B (IgG)	1,110,000	1,270,000	6,800	-99.1
	3A (BSA)	4,900,000	6,210,000	4,050,000	-17.3
10	3B (IgG)	5,460,000	6,560,000	1,410,000	-74.2
	4A (BSA)	39,800	71,500	43,500	+ 9.3
	4B (IgG)	38,800	59,300	192	-99.5
	5A (BSA)	3,600,000	3,600,000	2,600,000	-27.8
	5B (IGG)	1,100,000	1,100,000	603	-99.9
15	6A (BSA)	48,600	85,200	71,000	+46.1
	6B (IGG)	72,500	130,300	920	-98.7
	7A (BSA)	29,400	50,300	41,400	+40.8
	7B (IGG)	42,600	69,400	690	-98.4

20 An increase in resistance of about 10-100% normally occurs when the carbon films of Example 2 are exposed to water. At a channel width of 1 mm, no complete bridging occurs which might cause a decrease in resistance. However, resistive shunting embodiments according to the invention can successfully detect a binding reaction even

25 without metal overcoating if a narrow channel width (or shorter path) is used, and other reaction conditions are adjusted accordingly.

#### D. Ultrasound Procedure

30 A pair of BSA and IgG diagnostic elements were prepared according to the procedure of Example 2, part B, using a 2 hour incubation period. After 2 hours both surfaces were washed and dried. A high level of gold particle binding was observed microscopically for the IgG sample, and a moderate to low amount of binding was

35 observed for the BSA sample. Both samples were then immersed in distilled water and subjected to 1 minute of

treatment with a Virsonic 50 Virtis cell disrupter at its lowest power setting. The samples were then removed and reexamined. No difference in the amount of gold binding was observed for the IgG sample, whereas most of the previously observed gold particles on the BSA sample were removed by the treatment. In another experiment in which a similar ultrasonic treatment was used, a false positive sample (a BSA sample for which a drop in resistance was noted) of the type described in Example 1, part E, was converted to a negative sample.

### EXAMPLE 3

The sensitivity of the method of the invention was evaluated by determining a limiting amount of antibody required for an assay. Diagnostic elements were prepared by cutting conductive polyimide plastic (Kapton containing blended carbon) into 1 cm by 0.3 cm squares. The pieces were rinsed once in coating buffer (described above) and immersed overnight at room temperature in test tubes containing either 1% BSA in coating buffer or 5  $\mu$ g/ml rabbit IgG (the antigen). The resulting sample elements were then washed three times with PBS-Triton solution (described above) and placed on parafilm in petri dishes. A solution of goat anti-rabbit IgG antibody bound to gold particles, prepared according to the procedure of Example 1, Parts A and B, was serially diluted, and two drops at each dilution were applied to one BSA-coated sample and one IgG-coated sample. After 2 hours of incubation at room temperature, the samples were first washed with PBS-Triton solution, and then with distilled water. The samples were then allowed to dry, and the resistance of each sample was measured. Silver overcoating was then carried out for each sample using the procedure of Example 2, part C. The resistance of each sample was then measured, and the number of silver counts at each dilution was determined by x-ray analysis.

The results were as follows, wherein resistance values (R) are given in megaohms except where noted:

TABLE 3

	Sample/ Dilution	Starting R	R After Gold	R After Silver	Silver Counts
5	1A (BSA) None	1.55	2.02	1.46	-
	1B (IgG) None	1.48	1.72	112 ohms	2861
	2A (BSA) 1:2	1.52	2.11	1.28	-
	2B (IgG) 1:2	1.47	-	-	2187
10	3A (BSA) 1:4	1.52	1.94	1.67	-
	3B (IgG) 1:4	1.53	1.57	0.31	1723
	4A (BSA) 1:8	1.41	1.60	1.43	-
	4B (IgG) 1:8	1.41	1.57	0.54	1174
	5A (BSA) 1:16	1.40	1.58	1.41	-
15	5B (IgG) 1:16	1.49	1.57	0.62	748
	6A (BSA) 1:32	1.48	1.85	1.46	-
	6B (IgG) 1:32	1.47	1.82	1.06	699

Resistance values after silver overcoating and silver counts obtained in this example are plotted against gold-antibody conjugate dilution in Figure 13, wherein X's represent silver counts and circles represent resistance in megaohms. The limiting antibody concentration was found to occur at a dilution of about 1:8. The resistance and silver count values both varied substantially linearly with concentration, and the change in resistance was found to vary inversely with the number of silver counts, demonstrating that the extent of resistive shunting was directly related to the number of silver overcoated aggregates bound to the surface.

## EXAMPLE 4

The sensitivity of the method of the invention was further evaluated by generating a dose response curve. Diagnostic elements were prepared by cutting  
5 conductive Kapton plastic as described in Example 3 into 2.3 cm by 0.3 cm pieces. Titanium metal contact pads (0.5 by 0.3 cm) were disposed at opposite ends of each strip 1.3 cm apart to form resistors. The resistance of  
10 each resistor was then measured. The resistors were rinsed once with coating buffer, incubated in rabbit IgG, and washed three times using the procedure of Example 3. Dilutions of rabbit IgG in coating buffer containing  
15 rabbit IgG in the amounts given in Table 4 below were prepared and preincubated with gold-goat anti-rabbit IgG conjugate prepared according to Example 3 at a 1:8 dilution for 11.5 minutes. The preincubated conjugate was then applied to the sample resistors disposed on  
20 parafilm in petri dishes and allowed to incubate for 2 hours at room temperature. The samples were then washed once with PBS-Triton solution and once with distilled water, and allowed to dry. The resistance of each sample  
25 was then measured by connecting an ohmmeter to each of the contact pads. The samples were then silver overcoated as described in Example 2, part C, and the resistance of the samples was again measured.

The results were as follows, wherein the resistance values (R) are expressed in kilohms and the amount of rabbit IgG (antigen) is expressed in nanograms:

-30-

TABLE 4

		Rab. IgG		R After	R After	%
	<u>Sample</u>	<u>Amount</u>	<u>Starting R</u>	<u>Gold</u>	<u>Silver</u>	<u>Change</u>
5	1A	1000	311	349	335	- 4.0
	1B	1000	318	355	340	- 4.2
	2A	500	391	410	395	- 3.7
	2B	500	347	375	351	- 6.4
	3A	100	347	383	344	-10.2
	3B	100	350	410	326	-20.5
10	4A	50	371	406	371	- 8.6
	4B	50	376	416	385	- 7.5
	5A	10	388	434	368	-15.2
	5B	10	370	444	399	-10.1
	6A	5	437	515	443	-14.0
	6B	5	394	498	394	-20.9
15	7A	1	418	498	178	-64.3
	7B	1	392	480	99	-79.4

Figure 14 graphically depicts a plot of percent of maximum resistance (100% minus percent change from Table 5) versus the amount of competing antigen, using the average of the two results A,B obtained for each amount of antigen plotted. The results indicate that the method of this example was sensitive enough to detect as little as 1 nanogram of antigen in a sample.



**CLAIMS:**

1. In a method of detecting a substance in a test sample, which substance is one of a pair of substances that undergo a specific binding reaction with each other, including the steps of reacting said test  
5 sample with a a reagent comprising the other of said pair of substances and determining the extent of reaction therebetween, the improvement which comprises:

contacting particles having a first one of said pair of substances bound to the surfaces thereof with a  
10 layer of the second of said pair of substances which substantially spans a path between a pair of spaced-apart electrical conductors superposed on a substantially non-electrically conductive base, each of said conductors being connected to means forming an electrical circuit  
15 which includes said conductors and said path therein, the binding reaction between said first and second substances causing said particles to be bound to said bottom wall to form aggregates;

removing unbound particles;  
20 coating exposed outer surfaces of said aggregates with an electrically conductive substance; and

measuring a change in the electrical current flow through said circuit caused by formation of said  
25 coated aggregates on said path, said electrical change indicating the level of said substance to be detected in said test sample.

2. The method of claim 1, wherein said step of measuring a change in current flow further comprises measuring a change in the electrical resistance across said electrical circuit.

3. The method of claim 1, wherein said particles are gold particles, and said of coating said aggregates further comprises treating said aggregates with a silver compound-containing coating reagent, treating the silver coated aggregates with an acid solution, fixing the silver coating, and rinsing the coated aggregates.

4. The method of claim 1, wherein said step of removing unbound particles further comprises ultrasonically treating non-specifically bound particles and washing said path.

5. A method of detecting a substance in a test sample, which substance is one of a pair of substances that undergo a specific binding reaction with each other, comprising the steps of:

(1) mixing, in a liquid medium, electrically conductive particles, said particles having a first one of said pair of substances bound to the surfaces thereof, with said test sample containing an unknown quantity of the second of said pair of substances, under conditions effective to cause binding of said second substance in said test sample to said first substance on the surfaces of said particles;

(2) disposing said particles on a substantially continuous layer of said second substance bound to an essentially non-conductive base to form aggregates of said particles bound to said layer of said second substance, said layer of said second substance substantially spanning the width of a channel between a pair of side-by-side, spaced apart conductive layers superposed on said non-conductive base, each of said conductive layers being connected to means defining an electrical circuit including said conductive layers and said channel;

25 (3) washing away excess unbound electrically  
conductive particles from said layer of said second  
substance;

(4) coating exposed outer surfaces of said  
aggregates with an electrically conductive substance  
which adheres selectively thereto; and

30 (5) measuring a change in current flow through  
said circuit caused by the presence of said aggregates in  
said channel.

6. A method of detecting a substance in a test  
sample, which substance is one of a pair of substances  
that undergo a specific binding reaction with each other,  
comprising the steps of:

5 (1) mixing said test sample containing an  
unknown quantity of a first one of said pair of  
substances with electrically conductive particles in a  
liquid medium, under conditions effective to cause  
binding of said first substance in said test sample to  
10 the surfaces of said particles;

(2) disposing said particles on a substantially  
continuous layer of the second of said substances bound  
to an essentially non-conductive base to form aggregates  
of said particles bound to said layer of said second  
15 substance, said layer of said second substance  
substantially spanning the width of a channel between a  
pair of side-by-side, spaced apart conductors superposed  
on said non-conductive base, each of said conductors  
being connected to means defining an electrical circuit  
20 including said conductive layers and said channel;

(3) washing away excess unbound electrically  
conductive particles from said layer of said second  
substance;

25 (4) coating exposed outer surfaces of said  
aggregates with an electrically conductive substance  
which adheres selectively thereto; and

(5) measuring a change in current flow through said circuit caused by the presence of said aggregates in said channel.

7. A diagnostic element for use in detecting a reaction between a pair of substances which specifically bind together, comprising:

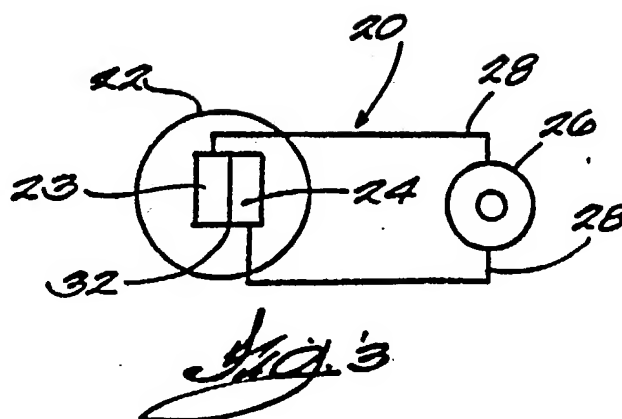
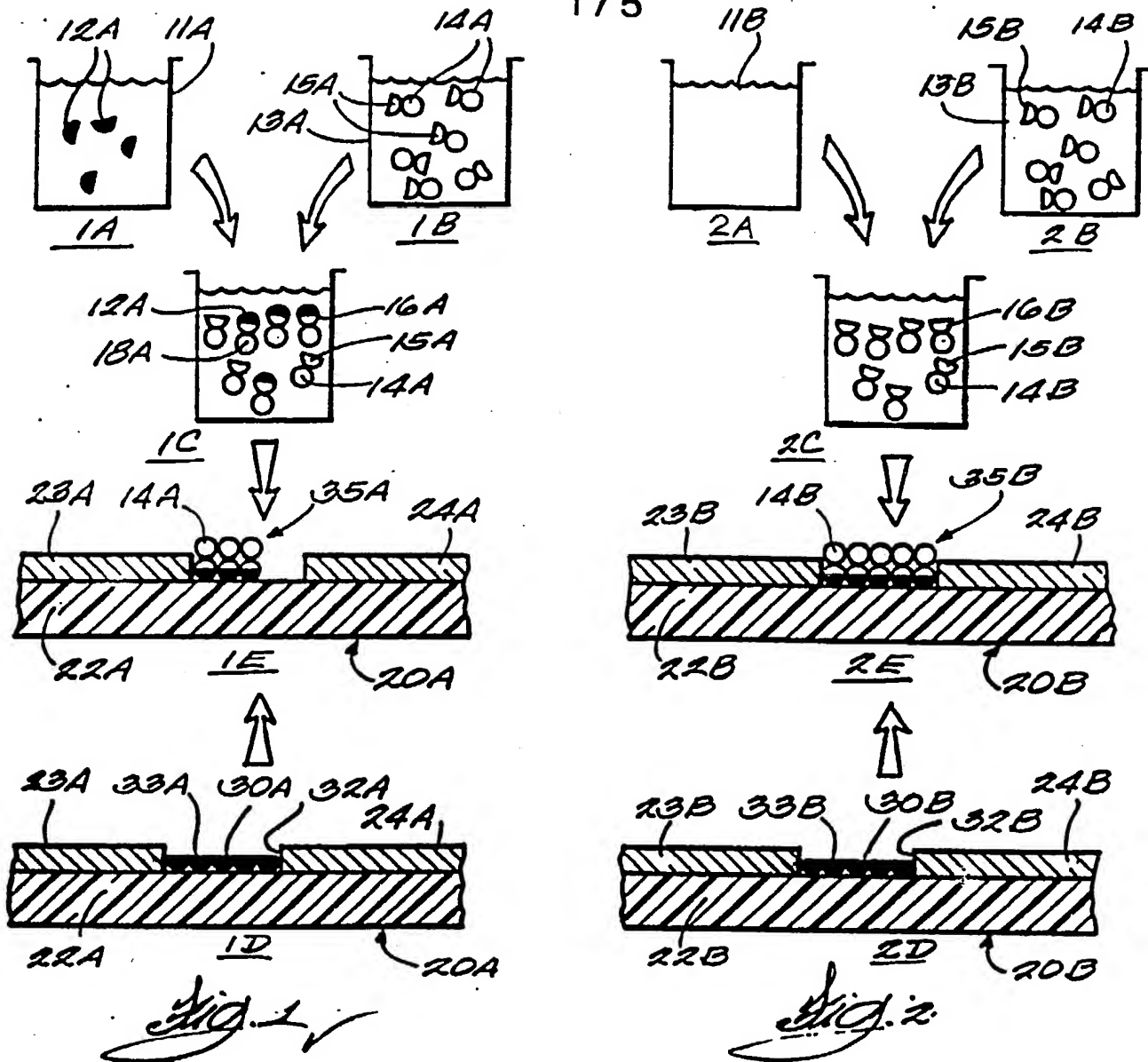
- 5 a electrically resistive support comprising bioreactive particles blended in a plastic material;
- a pair of spaced-apart electrical conductors superposed on said support; and
- 10 a layer of one of said pairs of substances disposed on said support along a path which spans said conductors.

8. The diagnostic element of claim 7, wherein said particles are made of carbon.

9. The diagnostic element of claim 7, wherein said plastic is polyimide.

10. The diagnostic element of claim 7, wherein said layer of said substance is selected from the group consisting of microorganisms or portions thereof, hormones, allergens, tumor markers, enzymes, steroids,  
5 and nucleotides.

1/5



SUBSTITUTE SHEET

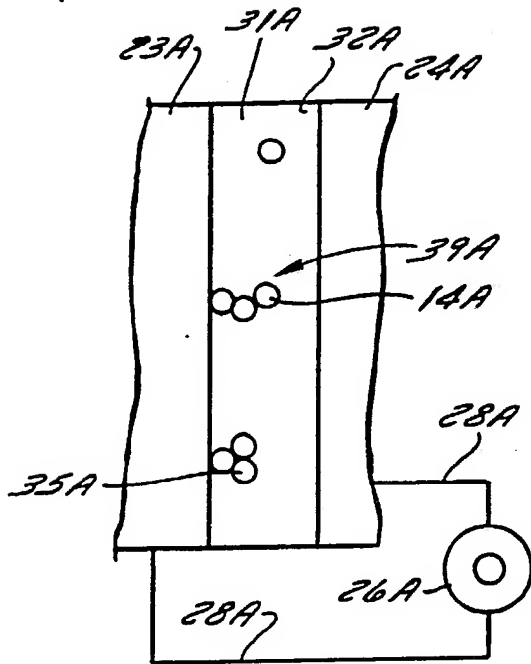


FIG. 4A

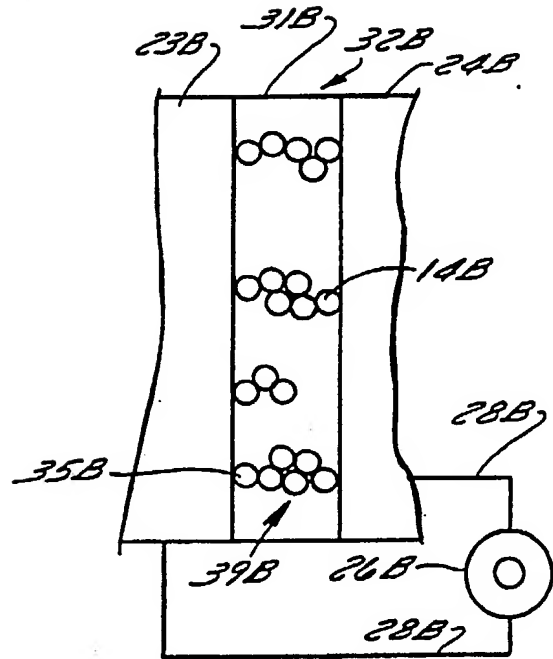


FIG. 4B

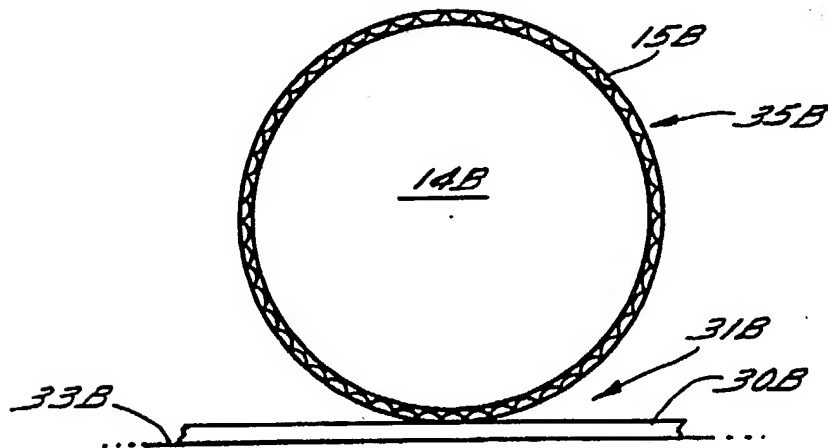


FIG. 5

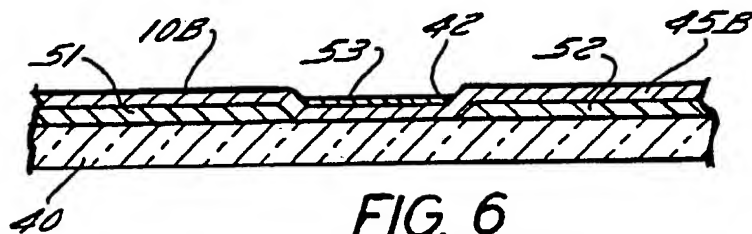
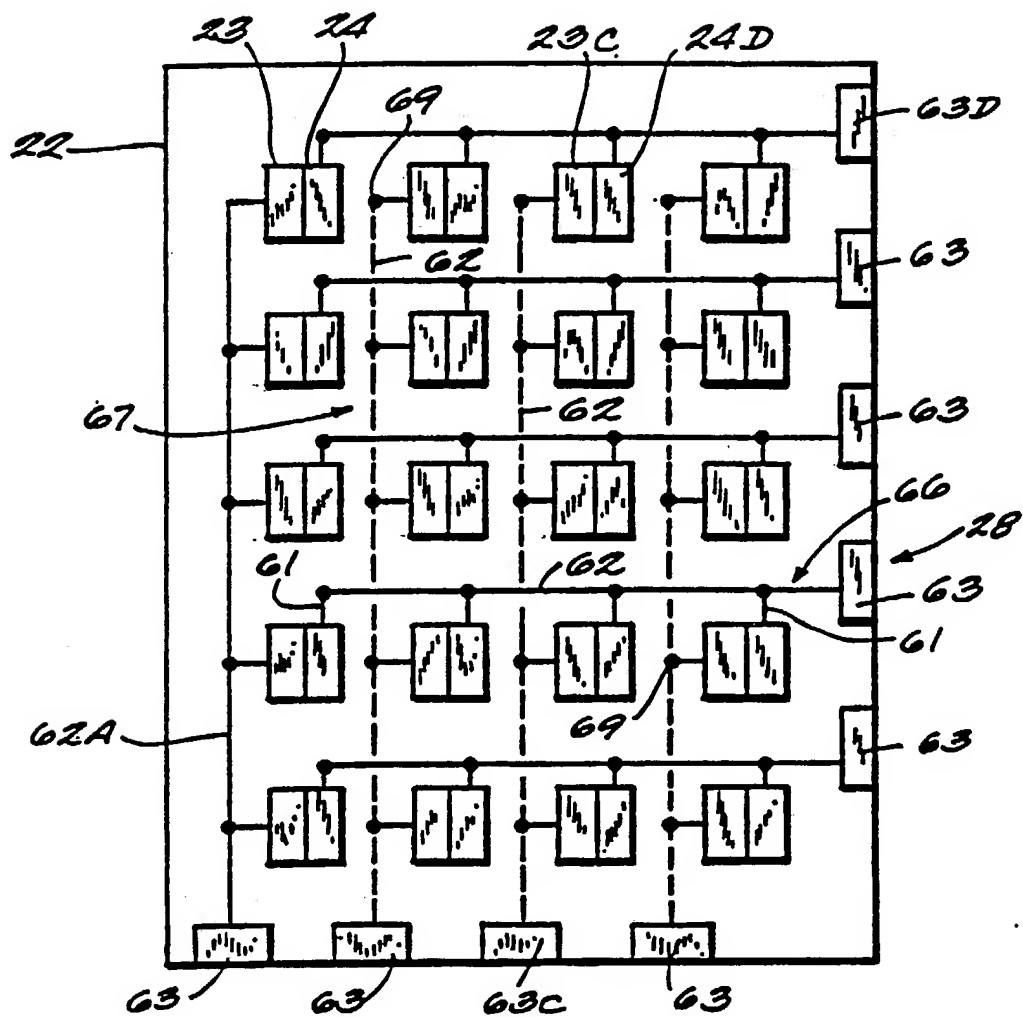
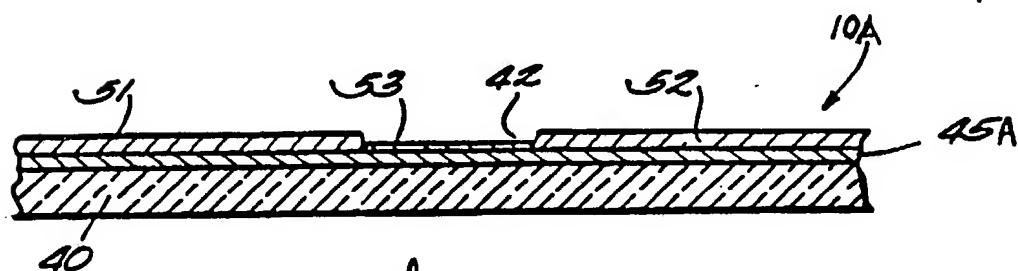
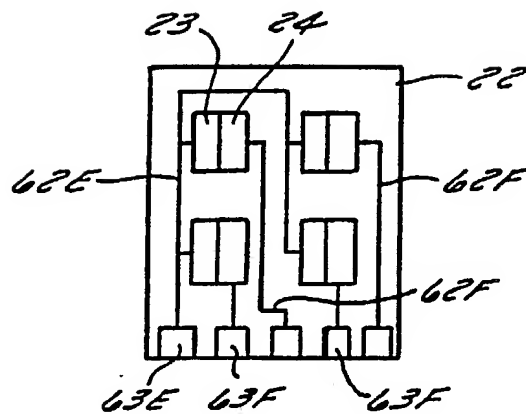
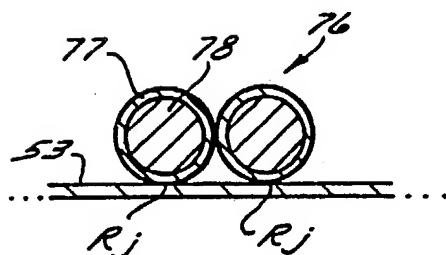
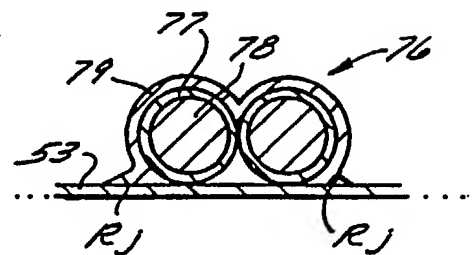
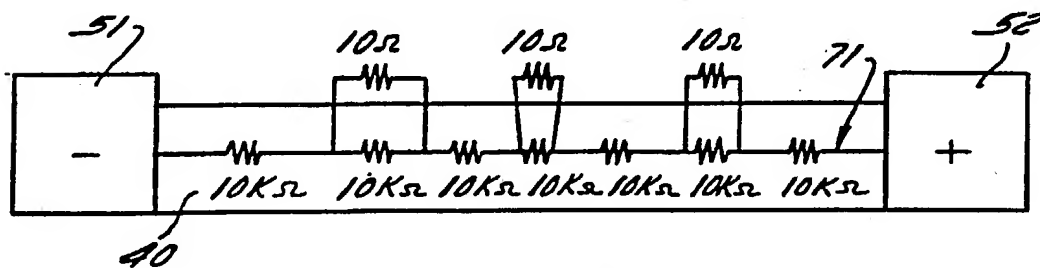
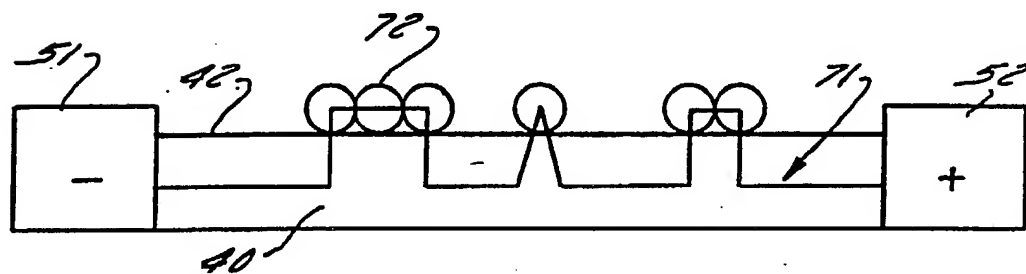


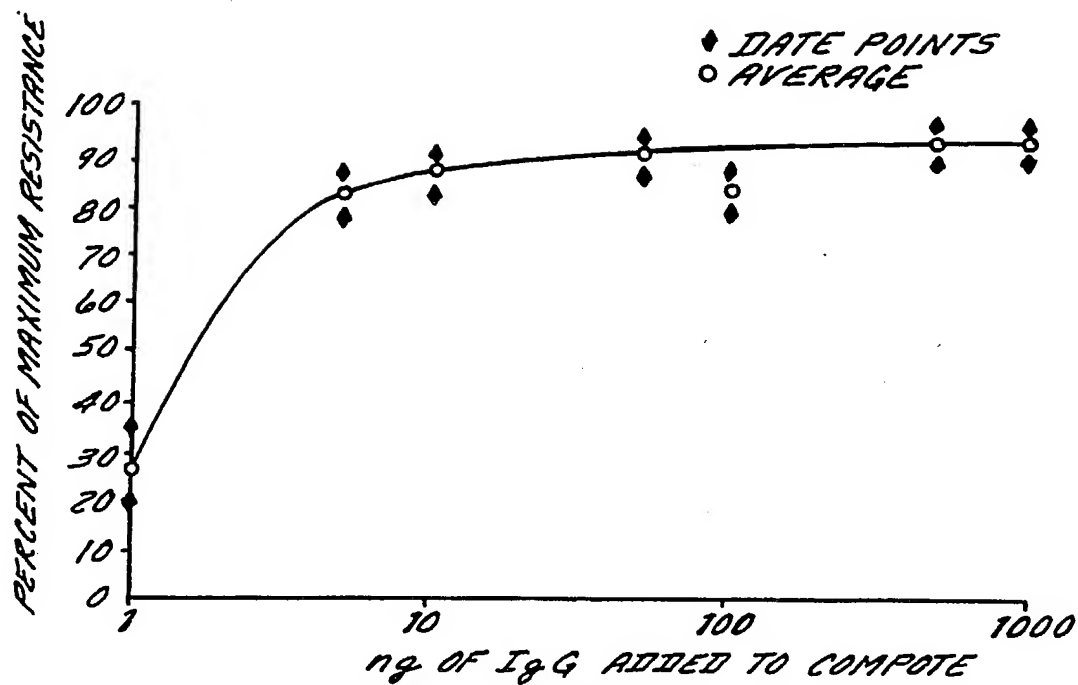
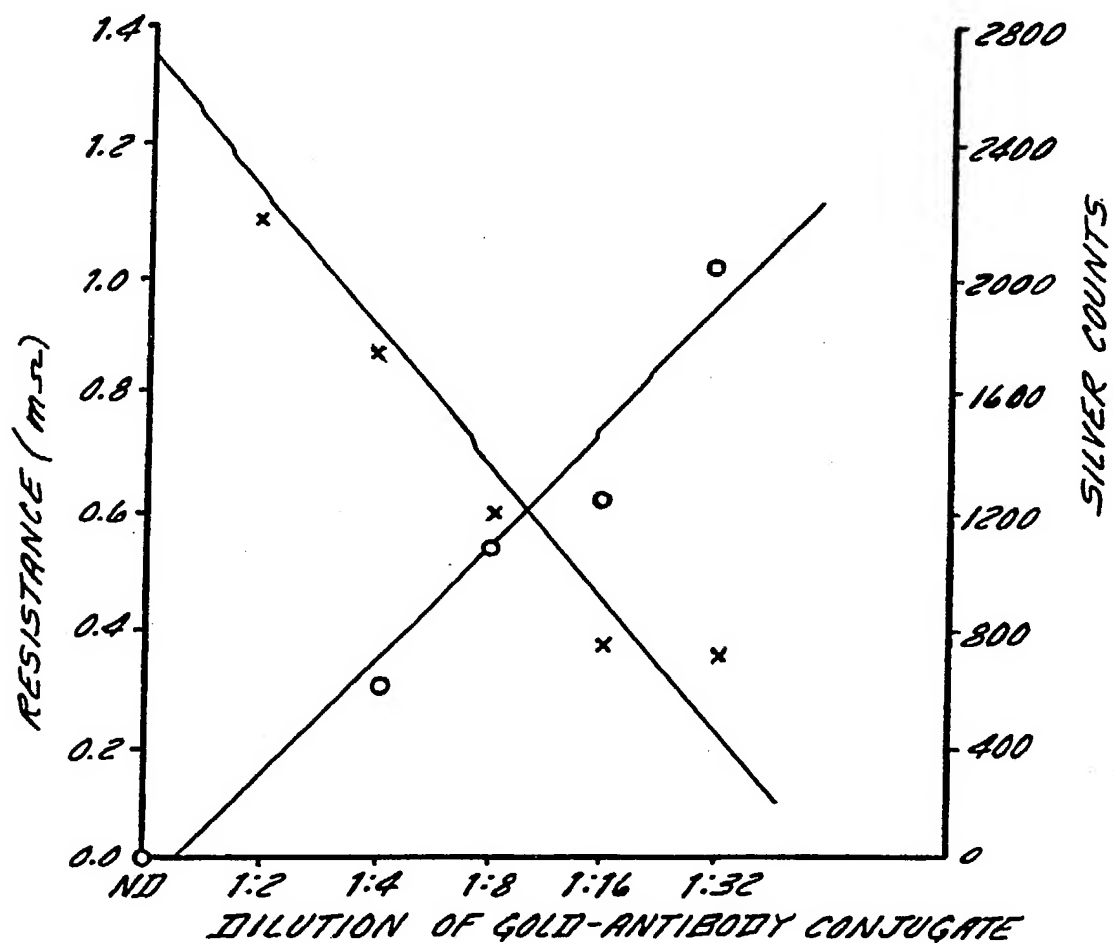
FIG. 6

*Fig. 8**Fig. 7*

SUBSTITUTE SHEET

FIG. 9FIG. 10FIG. 11FIG. 12



FIG. 14FIG. 13

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US89/05028

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): G01N 27/48, 27/00, 33/553, 33/58 U.S. Cl.: 436/501/518, 531, 435/7		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S. Cl.	435/7 436/501, 518, 531, 806, 807, 809	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
CAS, BIOSIS, APS DATABASES		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y, P, &	US, A, 4,794,089 (MROCZKOWSKI) 27 December 1988 (27.12.88) (Note: entire document).	1-10
Y, P	WO, A, 88/09499 (THE JOHNS HOPKINS UNIVERSITY) 1 December 1988 (01.12.88) (Note: page 5, line 7-page 6, line 33; claims 1-16; and Fig. 1a-2b, 5a-5c).	1-10
Y	US, A, 4,778,767 (HUMMELEN) 18 October 1988 (18.10.88) (Note: abstract; column 2, line 43-column 3, line 23; column 3, lines 39-44).	7, 9, 10
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
17 January 1990	13 FEB 1990	
International Searching Authority	Signature of Authorized Officer	
ISA/US	CAROL A. SPIEGEL <i>Carol A. Spiegel</i>	

## III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,775,636 (MOEREMANS) 4 October 1988 (04.10.88) (Note: column 6, lines 25-66; column 13, lines 16-22; claims 13-14).	1-6
Y	US, A, 4,219,335 (EBERSOLE) 26 August 1980 (26.08.80) (Note: abstract, Fig. 2, column 2, lines 30-65; column 5, line 29-column 6, line 17; claims 1,3-5,7-10).	1-10
A	US, A, 4,777,019 (DANDEKAR) 11 October 1988 (11.10.88) (Note: entire document).	1-10
A	US, A, 4,713,347 (MITCHELL) 15 December 1987 (15.12.87) (Note: entire document).	1-10